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Mouse Lung Structure and Function after Long-Term Exposure to an Atmospheric Carbon Dioxide Level Predicted by Climate Change Modeling

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Background: Climate change models predict that atmospheric carbon dioxide [CO_2] levels will be between 700 and 900 ppm within the next 80 y. Despite this, the direct physiological effects of exposure to slightly elevated atmospheric CO_2 (as compared with ∼410 ppm experienced today), especially when exposures extend from preconception to adulthood, have not been thoroughly studied.

Objectives: In this study we aimed to assess the respiratory structure and function effects of long-term exposure to 890 ppm CO_2 from preconception to adulthood using a mouse model.

Methods: We exposed mice to CO_2 (∼890 ppm) from prepregnancy, through the *in utero* and early life periods, until 3 months of age, at which point we assessed respiratory function using the forced oscillation technique, and lung structure.

Results: CO_2 exposure resulted in a range of respiratory impairments, particularly in female mice, including higher tissue elastance, longer chord length, and lower lung compliance. Importantly, we also assessed the lung function of the dams that gave birth to our experimental subjects. Even though these mice had been exposed to the same level of increased CO_2 for a similar amount of time (∼8 wk), we measured no impairments in lung function. This suggests that the early life period, when lungs are undergoing rapid growth and development, is particularly sensitive to CO_2.

Discussion: To the best of our knowledge, this study, for the first time, shows that long-term exposure to environmentally relevant levels of CO_2 can impact respiratory function in the mouse. https://doi.org/10.1289/EHP7305

Introduction

Throughout human evolution, atmospheric carbon dioxide (CO_2) levels have been close to 250 ppm (Hönisch et al. 2009). Today, greenhouse gas emissions due to human activities are driving global climate change, with CO_2 being a major contributor (IPCC 2013). In 2014, the level of CO_2 in the atmosphere (as derived from *in situ* air measurements at Mauna Loa Observatory, Hawaii) crossed the 400 ppm threshold for the first time (Keeling and Keeling 2017), meaning that we are now constantly exposed to levels higher than humans have ever previously experienced. Climate change models include probable scenarios where atmospheric CO_2 in 2100 may approach 700 ppm [Representative Concentration Pathway (RCP) 6.0] or even exceed 900 ppm (RCP 8.5) (USGCRP 2017). Of critical importance, few studies have focused on the direct health effects of long-term exposure to increased atmospheric CO_2 (when compared with current levels) and the urgent need to address this lack of knowledge has recently been highlighted (Jacobson et al. 2019).

Although the acute, toxic effects of exposure to very high levels of CO_2 (i.e., >100,000 ppm) are well known (Langford 2005; Scott et al. 2009), the potential impacts of prolonged exposure to levels predicted by climate change models are vague. The sparse research in this broad area includes studies aimed at understanding the effects of exposure to elevated CO_2 in poorly ventilated classrooms/offices (Bakó-Biró et al. 2012; Lu et al. 2015; Muscatiello et al. 2015), submarines (Rodeheffer et al. 2018), and spacecraft (Law et al. 2010; James et al. 2011; Cronyn et al. 2012; Hughson et al. 2016) as well as small mammal laboratory studies aimed at understanding CO_2-induced physiological changes (Schaefer 1982; Wade et al. 2000; Kiray et al. 2014; Martrette et al. 2017). Many of these studies have been succinctly summarized in a recent review (Jacobson et al. 2019).

Importantly, the vast majority of previous research in this area exposes humans or experimental animals (generally mice, rats, or guinea pigs) to levels of CO_2 in excess of that predicted by climate change models (i.e., >1,000 ppm) for relatively short durations (i.e., hours to a few months). They do not extend to the *in utero*/early life periods when lung growth and development are rapidly occurring. Furthermore, controlled human exposure studies and laboratory studies are generally conducted in young, healthy adults who are likely to be the most able to cope with such exposure (Rice 2004). These previous studies show that exposure to CO_2 between 1,000 and 5,000 ppm for hours to months can result in a range of adverse health effects, including inflammation (Schneberger et al. 2017; Thom et al. 2017a, 2017b) and cognitive impairment (Kajtár and Herczeg 2012; Satish et al. 2012; Allen et al. 2016; Snow et al. 2019), with some effects manifesting after as little as a few hours of exposure. As the duration of exposure increases to weeks or months, kidney calcification, bone demineralization (Schaefer et al. 1979; Schaefer 1982) and a range of behavioral changes have been measured (Kiray et al. 2014; Martrette et al. 2017).

To the best of our knowledge, there are no previous studies in which experimental subjects were exposed from preconception to adulthood to the levels of CO_2 that are realistically possible in the near future (i.e., 600 to 900 ppm). This context is critical for the multifactorial implications of the current CO_2 emission rates continuing. Here, we establish the first mammalian model of long-term exposure to ambient CO_2 at a level predicted by climate modeling that incorporates the period from preconception to adulthood. We continually exposed mice for this period to ∼890 ppm CO_2 and then assessed their respiratory structure and function. We focused on respiratory structure and function given that the lungs are the initial site for CO_2 exposure and that they

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play a critical role in maintaining the body's acid-base balance (Seifter and Chang 2017). It is thus likely that if any organ is going to be impacted by moderately increased CO_2, it would be the lungs. We also assessed the lung function of the dams, which were exposed to $\sim$890 ppm CO_2 for approximately the same duration, although they started exposure as adults.

Methods

Animals and Exposures

Adult (8-wk old) C57BL/6 mice were purchased from the Animal Resources Center (Murdoch, WA, Australia) and housed at the Telethon Kids Institute under a 12-h light:dark cycle, in a room held at 23°C. Food (Rat and Mouse Cubes; Speciality Feeds) and water were available *ad libitum*. Mice were housed individually in cages with standard open metal grill lids to allow air circulation, in addition to being provided with an $\sim$10-cm length of polyvinyl chloride pipe and cotton nestlet material for bedding/nesting (Able Scientific). We used aspen wood chips as the cage substrate, and the cages were changed at least once per week throughout the experiment. This type of substrate and husbandry regime has been shown to be more than sufficient to keep ammonia to almost negligible concentrations in static cages (Ferrecchia et al. 2014). Twenty female and 10 stud male mice were randomly assigned to two experimental conditions; female control mice (Con) were housed at 465 ± 27 ppm atmospheric CO_2, and female experimental mice (CO_2 exposure) were housed at 889 ± 123 ppm atmospheric CO_2 (predicted atmospheric conditions). Stud males were housed individually under control conditions except for over-night mating. Ten female mice were allocated to each treatment. Mice in the CO_2-exposed group were housed within a semi-sealed, custom-made 393-L exposure chamber with internal dimensions of $60 \times 50 \times 140$ cm (L × W × H). Acknowledging that CO_2 is heavier than air, the exposure chamber was also fitted with two 80-mm fans that ran continuously to circulate air in the chamber. In addition, the position of the individual boxes within the chamber was rotated every 3 d to ensure an even exposure for each mouse. Elevated CO_2 was maintained using a ProCO$_2$ P120 (BioSpherix Ltd.). The ProCO$_2$ P120 works by continuously monitoring the level of CO_2 in the chamber (using a sensor placed at the chamber midpoint—within the breathing zone of the experimental animals) and when a predetermined set point is reached (in this study, 900 ppm CO_2), medical air briefly flows into the chamber from a regulated source. Chamber CO_2 rises due to the natural respiration of the animals housed inside and is lowered again by the inflowing medical air. In this way, chamber CO_2 was maintained at 889 ± 123 ppm. CO_2 levels in the chamber were recorded by taking remote snapshots of the ProCO$_2$ P120 digital readout every 10 min for the duration of the study. This resulted in almost 12,000 images from which we randomly selected one per hour to manually record and average the CO_2 level (creating 2,832 recordings over the 118-d exposure period). Mice in the control group were housed in the same type of cages in the same room, but not within the exposure chamber. Thus, they were exposed to the background atmospheric CO_2 of the room; this level of CO_2 was not regulated. The CO_2 level of the room where the Con mice were held was also measured using the ProCO$_2$ P120, with 90 measurements taken at random intervals over the 118-d exposure period. This level of sampling was sufficient given that the CO_2 level of the room was very stable (465 ± 27 ppm).

Following $\sim$1 wk of acclimatization, the female mice were assessed daily to estimate their estrous cycle stage (Caligioni 2009). This involved visual inspection of the vaginal opening. Pink, swollen vaginal openings were classified as fertile. Females identified as being in the correct stage of the estrous cycle were placed with a stud male overnight. Only pair-matings (1 male to 1 female) were performed. Mated females were then housed singly throughout pregnancy and lactation in their assigned atmospheric condition (Figure 1). Pups were weaned at postnatal day (PND) 28 and then group housed with their littermates by sex (2–5 animals per box) in their assigned atmospheric condition until use in experiments. We had a total of 53 pups (23 male and 30 female) in the CO_2 treatment, born from eight litters, and 38 pups (19 male and 19 female) in the control group, born from six litters. For all analyses, we treated individual pups as a single *n*. When the aforementioned pups were weaned, the remaining dams also had their lung function assessed before being euthanized. All studies were performed according to animal health and welfare guidelines and were approved by the Telethon Kids Institute Animal Ethics Committee (AEC#324).

Respiratory Function Assessments

At 12 wk of age (or $\sim$16 wk of age for dams), the mice were weighed and anesthetized in preparation for lung function assessment by intraperitoneal injection of a mixture containing xylazine (1 mg/mL; Troy Laboratories) and ketamine (20 mg/mL; Troy Laboratories) at a dose of 0.1 mL/10 g body weight. Once surgical anesthesia was achieved, the mice were tracheostomized with a 10-mm length of polyethylene cannula (internal diameter = 0.086 mm), which was secured with surgical silk. Mice were then placed inside a plethysmograph and ventilated at 400 breaths/min with a tidal volume of 8 mL/kg and a positive end expiratory pressure of 2 cm water (H_2O) (Minivent; Harvard). This ventilation regime was sufficient to suppress spontaneous breathing efforts and thus allow measurement of thoracic gas volume (TGV) and lung mechanics without paralysis (Larcombe et al. 2011).

TGV and lung function at functional residual capacity (FRC) were measured as previously described (Sly et al. 2003; Jánosi et al. 2006). Briefly, to measure TGV, two small electrodes were inserted into the intercostal muscles. During a 6-s apneic period, the plethysmograph and airway were occluded and the muscles electrically stimulated with pulses of $\sim$20 V that were $\sim$1–2 ms in duration. TGV was calculated by applying Boyle's law to the relationship between plethysmograph and tracheal pressures, which were measured using transducers (Validyne MP45; Validyne Engineering; and model 8507C-2; Endevco, respectively). The equation for calculating TGV used was derived from Jánosi et al. (2006) as $TGV = -s\beta(V_{box} - V_m) - V_{ds}$, where s is the slope of the regression line given by the change in pressure in the plethysmograph/change in pressure at the trachea; β is the atmospheric pressure minus partial pressure of water vapor in the

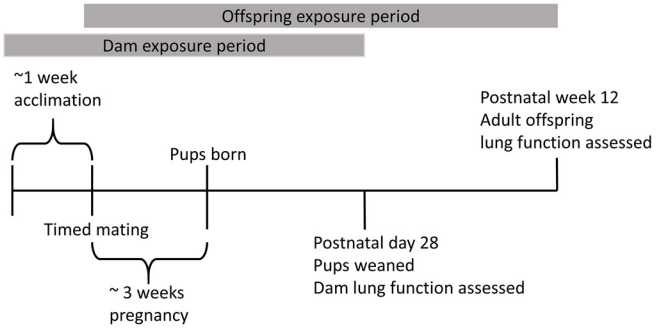

Figure 1. Schematic representation of the study design. Adult mice were acclimated to the elevated CO_2 (or control) for $\sim$1 wk prior to timed mating. They remained in their assigned CO_2 concentration until pups were weaned on postnatal day 28, at which point dams had their lung function assessed and samples were taken. Weaned offspring stayed in their assigned CO_2 concentration until they were 12 weeks of age, at which point their lung function was assessed and samples taken. Note: CO_2, carbon dioxide.

alveolar gas (DuBois et al. 1956) divided by the atmospheric pressure; V_{box} is the volume of gas in the plethysmograph; V_m is the volume occupied by the mouse in the plethysmograph; and V_{ds} is the instrumental dead space between the tracheal cannula and the site of occlusion (in this study, the $V_{ds} = 0.05$ mL).

Respiratory system impedance (Z_{rs}) was measured at FRC using a small-animal wave tube modification of the forced oscillation technique (FOT) (Fredberg and Stamenovic 1989; Sly et al. 2003). During 6-s apneic periods, a FOT signal was delivered via a 100-cm long wave tube to the tracheostomized mouse. Pressure was measured at either end of the wave tube (8507C-2; Endevco), and Z_{rs} was estimated as the load-impedance on the wave tube. Z_{rs} was partitioned into components representing the airways [airway resistance (R_{aw})] and lung parenchyma [tissue damping (G), and tissue elastance (H)] using the constant phase model (Hantos et al. 1992). Hysteresivity (η) was calculated as G/H (Fredberg and Stamenovic 1989).

Pressure–volume (PV) curves between the FRC and 20-cm water (H_2O) transrespiratory pressure were generated by evacuating the sealed plethysmograph with a regulated vacuum source. From the deflationary part of the PV curves, we calculated compliance as $(V8 - V3)/5$, where V is lung volume at a given pressure (Limjunyawong et al. 2015).

Biological Sample Collection and Processing

Following completion of respiratory function testing, blood was immediately obtained from a randomly selected subset of mice (8–10 per sex per treatment) by cardiac puncture and ~100-μL aliquots were taken from each sample for i-STAT (Abbot) measurement of blood pH. Bronchoalveolar lavage (BAL) fluid was then collected from mice via washing 0.5 mL of saline in and out of the lungs three times. This sample was processed for total and differential cell counts as previously described (Larcombe et al. 2011). The total number of cells was estimated using a hemocytometer. Differential cell counts were performed on a randomly selected subsample of six mice from each sex for each treatment. For these mice, cytospin slides were stained with Diff-Quik™ stain set 64851 (Labs Aids) as per the manufacturer's protocols. Differential cell counts for macrophages, neutrophils, and eosinophils were performed using light microscopy by a member of the team who was blinded to treatment. Total protein content in BAL fluid was measured using a colorimetric assay (Bio-Rad) (Bradford 1976) for 13–17 randomly selected mice from each sex for each treatment. We inflation-fixed the lungs of a randomly selected subsample of 11–14 mice from each sex for each treatment (Hsia et al. 2010) prior to removal *en bloc*. The left lung was embedded in paraffin, and 5-μm thick sections were taken from the proximal region. One section from each mouse was stained using Masson's trichome for semiautomated assessment of chord length (Crowley et al. 2019) and collagen. Sections were imaged using a Panoramic MIDI® scanner (3DHISTECH Ltd.), and collagen content was quantified as a percentage of total tissue in the cross-sectional area using ImageJ (Schneider et al. 2012). Additional 5-μm thick sections were obtained from 5–6 randomly selected individuals from each sex for each treatment. They were stained with hematoxylin and eosin and examined under light microscopy. Stereological software (newCAST™; Visiopharm) and point counts were used to calculate parenchymal volume (V_p), the volume of the alveolar septa (V_s), and volume of air in the parenchyma ($V_{a(p)}$). The number of alveoli ($N_{(a,L)}$) was calculated using the volume dissector method, which allows alveoli to be unbiasedly counted in a virtual three-dimensional volume generated from two consecutive sections separated by a known distance (Sterio 1984; Hsia et al. 2010).

Statistical Analyses

Statistical analyses were performed using SigmaPlot (version 14; Systat Software). Two-way analysis of variance (ANOVA), with factors of treatment group and sex was used to assess for significant differences between groups. For all ANOVAs, $p < 0.05$ was considered significant and the Holm-Sidak post hoc test was used to analyze differences between groups. For lung structure and function outcomes for dams, t-tests were used to assess for significant differences between control and CO_2-exposed mice. Data are expressed as means ± standard deviations (SDs).

Results

Thoracic Gas Volume

There was no significant effect of treatment on TGV for either sex ($p = 0.251$ for females and $p = 0.299$ for males; Figure 2A); however, the lung volume of CO_2-exposed females (0.276 ± 0.073 mL) was 112.5% of that of Con females (0.246 ± 0.069 mL). Similarly, the lung volume of CO_2-exposed males (0.288 ± 0.079 mL) was 110% of that of Con males (0.262 ± 0.058 mL). Given this consistent trend, we present specific airway resistance (sR_{aw}), tissue damping (sG), and tissue elastance (sH).

Lung Function at FRC

There was no statistically significant effect of treatment for either sR_{aw} ($p = 0.193$ for females and $p = 0.543$ for males) or sG ($p = 0.142$ for females and $p = 0.235$ for males) for either sex (Figure 2B,C). There were common themes for both parameters, with potentially biologically significant differences in sG being observed for both male ($CO_2 = 113.4\%$ of Con) and female ($CO_2 = 115.8\%$ of Con) mice. The sR_{aw} of CO_2-exposed females was also 115.3% of that of Con females. There was a statistically significant effect of CO_2 exposure on sH for female ($p = 0.015$), but not male ($p = 0.370$) mice (Figure 2D). The sH of female CO_2-exposed mice was 129.5% of that of Con females. There was no statistically significant effect of treatment on hysteresivity for either sex ($p = 0.133$ for females and $p = 0.960$ for males).

Lung Compliance

CO_2 exposure resulted in significantly lower lung compliance in female mice ($p = 0.016$; Table 1). There was no effect of CO_2 exposure on lung compliance in male mice ($p = 0.626$).

Blood pH, Lung Inflammation, and Structure

The blood pH of CO_2-exposed mice was 0.05 to 0.07 lower than that of Con mice of the same sex (Table 1). These differences were not statistically significant ($p = 0.342$ for female mice and $p = 0.373$ for male mice). There was no effect of treatment or sex on the total number of cells in BAL fluid ($p = 0.686$; Table 1), and virtually all cells were macrophages, regardless of sex or treatment. There was no significant effect of CO_2 exposure on BAL protein content for either sex ($p = 0.071$ for females and $p = 0.099$ for males). Mean chord length was significantly higher in CO_2-exposed female mice compared with Con female mice ($p = 0.034$); there was no effect in male mice ($p = 0.538$; Table 1). The proportion of lung that was collagen was not significantly impacted by CO_2 exposure ($p = 0.858$) or sex ($p = 0.913$; Table 1). There was no statistically significant effect of treatment ($p = 0.775$) or sex ($p = 0.124$) for parenchymal volume, volume of the alveolar septa ($p = 0.171$ for treatment and $p = 0.861$ for sex), volume of air in the parenchyma ($p = 0.216$ for treatment and $p = 0.770$ for sex), or number of alveoli ($p = 0.910$ for treatment and $p = 0.916$ for sex). In CO_2-exposed female mice, the volume of alveolar septa

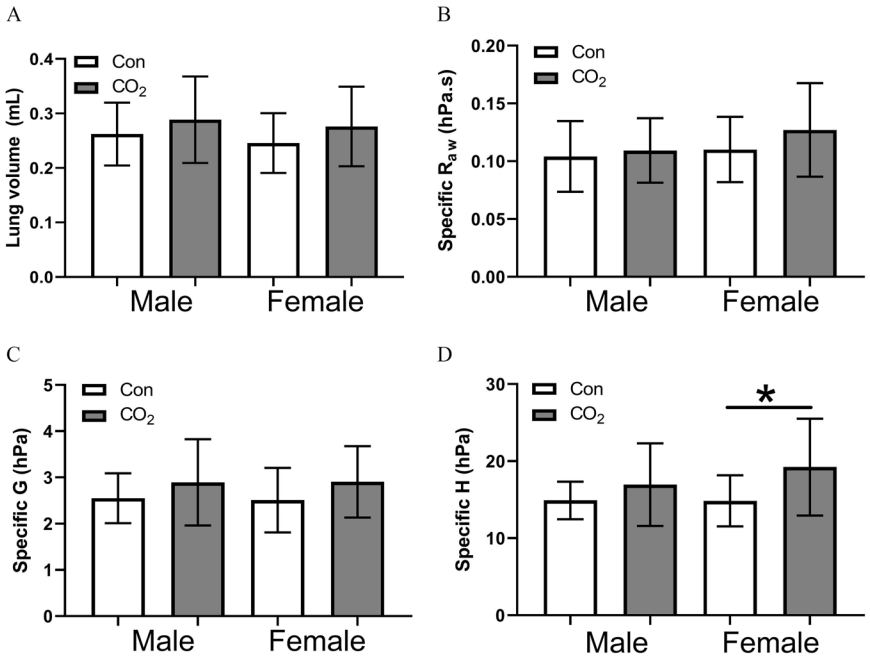

Figure 2. (A) Lung volume and lung function [(B) specific airway resistance, (C) specific tissue damping, and (D) specific tissue elastance] at functional residual capacity in male and female mice exposed to ~890 ppm CO_2 (gray bars) from preconception to adulthood or control (465 ppm; white bars). Data are expressed as means ± SDs. $n = 15–21$ per sex per treatment. *indicates significant effect of CO_2 on tissue elastance for female mice (2-way ANOVA). Summary data are available in Table S1. Note: ANOVA, analysis of variance; CO_2, carbon dioxide; SD, standard deviation.

was 20.8% lower, and the number of alveoli was 14.1% lower than in Con females. Similar differences were not observed in male mice (Table 1), which showed the opposite trends (i.e., 13.0% higher volume of alveolar septa and 12.8% more alveoli in CO_2-exposed male mice compared with Con males).

Lung Function of Dams

When the pups were weaned, we measured lung volume and function of the dams. Like the pups, these mice had been housed in conditions of either ~890 ppm CO_2 or 465 ppm CO_2; however, their exposure commenced when they were ~8 wk of age, continuing from ~1 wk preconception, throughout pregnancy, until the pups were weaned at PND28. This resulted in ~8 wk of exposure for the dams. We had six Con and six CO_2-exposed dams from which we obtained lung function data. There was no significant effect of CO_2 exposure on lung volume or on any parameter of lung function at FRC ($p > 0.55$ in all cases; Table 2) in these mice.

Discussion

This study showed that exposure to a CO_2 level of 890 ppm from preconception through to adulthood significantly impacted respiratory structure and function in female mice. Although the changes measured were modest, and only seen in female offspring, our data show exposure to CO_2 at a concentration of slightly more than double the current atmospheric levels [412 ppm; as derived from *in situ* air measurements at Mauna Loa Observatory, Hawaii (Keeling and Keeling 2017)] can have measurable functional effects. This was a somewhat surprising outcome because we would expect mice to be able to accommodate a modest increase in CO_2 without significant changes in respiratory structure or function. Moreover, the timing of exposure appeared to be important in that female mice (dams) which commenced exposure as adults and were exposed to the same level of CO_2 for approximately the same duration, showed no alterations in lung function. We specifically chose to expose experimental animals to a level of CO_2 that is realistic and expected to occur within current human lifetimes based on climate change models (USGCRP 2017). We also chose mice for this study because, even though they are likely more tolerant of exposure to elevated CO_2 due to their burrowing lifestyle (Studier and Baca 1968; Williams and Rausch 1973), we wanted to investigate the effects of exposure from *in utero* all the way to adulthood. This is only logistically possible in a species with a relatively short life span. The fact that we were able to measure CO_2-induced functional and structural changes in a species that is likely better physiological equipped to deal with increased CO_2 levels, suggests that the potential impacts on humans may be even more overt. We acknowledge that there are limitations with respect to using mice for a study such as this, including important differences in their lung developmental trajectories, the aforementioned increased tolerance of higher CO_2, and known respiratory structural differences between mice and humans. However, the benefits outweigh the limitations. We were able to expose mice from preconception to adulthood within a few months and then comprehensively assess their respiratory structure and function. This would be far more difficult in a larger/longer-lived species, and impossible in humans.

Mice that lived in the 890-ppm CO_2 environment during *in utero* development up to adulthood had slightly lower blood pH values compared with Con mice as adults (Table 1). Although this 0.05–0.07 lower pH was not statistically significant, it could reflect borderline respiratory acidosis in CO_2-exposed mice. Similar reductions in blood pH values have previously been reported in CO_2-exposed humans (Halperin 2007); however, those subjects (submariners) were exposed to much higher levels of CO_2 (~7,000 ppm) for shorter periods of time while operational. Importantly, long-term exposure to real-world atmospheric CO_2 levels has recently been shown to result in increased serum bicarbonate levels in a sample of 33,546 U.S. adults (Zheutlin et al. 2014). This increase of 6.3% occurred between 2000 and 2012 and paralleled the 6% increase in atmospheric CO_2 over the same period (from ~369 to ~392 ppm). A higher serum bicarbonate level

Table 1. Blood, bronchoalveolar lavage fluid, and lung structure parameters [means ± SDs (n)] in male and female mice exposed to ~890 ppm CO_2 from preconception to adulthood or control (465 ppm).

Measurement	Con male	CO_2 male	Con female	CO_2 female
Body weight	23.4 ± 0.3 (19)	23.1 ± 0.3 (23)	19.7 ± 0.1 (19)*	18.4 ± 0.2 (30)*
Blood pH	7.06 ± 0.03 (10)	7.01 ± 0.06 (9)	7.08 ± 0.02 (9)	7.01 ± 0.05 (8)
Total cells (*n*/mL)	53,155 ± 36,402 (19)	52,464 ± 29,147 (23)	40,604 ± 18,770 (18)	46,196 ± 25,698 (30)
Macrophages (%)	99.9 ± 0.1 (6)	99.9 ± 0.1 (6)	99.7 ± 0.4 (6)	98.3 ± 3.1 (6)
Protein (mg/mL)	0.24 ± 0.05 (13)	0.27 ± 0.09 (13)	0.25 ± 0.05 (14)	0.23 ± 0.03 (17)
Chord length (μm)	22.4 ± 1.3 (13)	22.1 ± 1.7 (14)	21.3 ± 1.3 (11)*	22.6 ± 1.5 (12)*
Compliance (mL/cm H_2O)	0.046 ± 0.004 (19)	0.047 ± 0.006 (23)	0.045 ± 0.006 (19)*	0.041 ± 0.006 (28)*
Collagen (% of total lung tissue)	5.78 ± 1.73 (13)	5.46 ± 1.47 (14)	5.56 ± 1.35 (11)	5.57 ± 1.31 (12)
Parenchymal volume (mm^3)	223.6 ± 51.6 (5)	250.2 ± 95.0 (6)	197.4 ± 46.7 (6)	186.8 ± 37.0 (6)
Volume of the alveolar septa (mm^3)	85.2 ± 18.6 (5)	96.3 ± 41.5 (6)	79.7 ± 17.5 (6)	63.2 ± 15.6 (6)
Volume of air in the parenchyma (mm^3)	125.9 ± 37.2 (5)	137.4 ± 52.7 (6)	114.7 ± 26.3 (6)	110.3 ± 27.7 (6)
Alveoli (*n*)	$2.74 \times 10^6 \pm 1.17 \times 10^6$ (5)	$3.10 \times 10^6 \pm 1.49 \times 10^6$ (6)	$3.20 \times 10^6 \pm 7.79 \times 10^5$ (6)	$2.75 \times 10^6 \pm 4.59 \times 10^5$ (6)

Note: ANOVA, analysis of variance; CO_2, carbon dioxide; Con, control; SD, standard deviation. *, significant effect of treatment within sex (2-way ANOVA).

is indicative of more CO_2 being stored in the body (Jacobson et al. 2019). Although the study by Zheutlin et al. (2014) did not report blood pH values, it presented clear evidence that a relatively minor increase in atmospheric CO_2 can have direct effects on blood chemistry, an outcome reflected in our results. Although this may sound counterintuitive given that the concentration of CO_2 in the human lung is ~56,000 ppm (Robertson 2001) and that in our study we increased that by only 425 ppm, Robertson (2001) eloquently described how an increase in atmospheric CO_2 by ~21% (353 in 2001 to a predicted 428.8 ppm in 2050) would reduce human blood pH values by ~0.08 units (from 7.400 to 7.319). This is "just outside the range of normal pH values of human blood and indicates the onset of acidosis" (Robertson 2001). Robertson (2001) speculated that in order to counter this change there would need to be an "evolutionary change in human metabolism" (Robertson 2001) or that "every human on the planet would have to continually and consciously deep breathe" (Robertson 2001). Clearly, neither of these are feasible, especially given that breathing is not consciously controlled. Furthermore, reductions in blood and tissue pH values are known to have downstream effects on a range of organs, including the lungs (Eckenhoff and Longnecker 1995). The normal physiological response to respiratory acidosis is to increase ventilation to blow-off excess CO_2 and increase blood pH values (Dean 2011). In our model, and in the future if climate change models are accurate, this tactic would be less effective owing to the higher ambient CO_2 level, presenting an ongoing and inescapable stressor to blood chemistry and lung function. In humans, increases in ventilation in response to respiratory acidosis are typically dominated by increases in tidal volume, rather than respiratory rate (Schaefer et al. 1963). Although we did not measure these parameters in our study, it is feasible that an extended period of increased tidal volume could impact lung structure and function.

Although, to the best of our knowledge, there are no other studies whereby experimental animals (or humans) have been exposed from preconception to adulthood to similar levels of CO_2 as in the present study, a very limited number of relatively old studies in humans did assess aspects of lung physiology after prolonged exposure to considerably higher levels of CO_2 (e.g., 8,500–20,000 ppm) for much shorter periods of time (e.g., 20–30 d) (Brown 1930; Schaefer et al. 1963; Gude and Schaefer 1969). A key finding of such studies is that lung dead-space volume can increase after CO_2 exposure. In these circumstances, a temporary increase in lung dead-space volume, as a proportion of tidal volume was observed, but this disappeared when the test subjects returned to a lower CO_2 environment. We did not specifically measure dead-space volume in our study, but mice exposed to 890-ppm CO_2 had a 10–12% higher TGV. It is feasible that this difference is, at least partially, a reflection of increased anatomical or alveolar dead space, which has been shown to be associated with changes in respiratory pattern (primarily an increase in tidal volume) (Schaefer et al. 1963).

Although we did not measure tidal volume, we observed a statistically significant greater chord length in female mice exposed to elevated CO_2 levels. Often, a greater chord length is associated with the destruction of alveolar walls in diseases such as chronic obstructive pulmonary disease (COPD) (Mitzner 2008). This is accompanied by increased lung volume and increased compliance. We did see a higher lung volume at FRC in female mice exposed to elevated CO_2 (consistent with obstructive lung disease), although these individuals also had a conflicting significantly lower compliance (Table 1), which is more a reflection of restrictive lung disease. The somewhat contradictory findings of higher chord length, coupled with higher *sH* and lower compliance strongly suggests there are multiple mechanisms at work.

In our opinion, it is extremely unlikely that exposure to elevated CO_2 causes alveolar destruction as seen in COPD; it is more likely that elevated CO_2 exposure is associated with impaired lung development, particularly in female mice. This is supported by our finding that the body weight of female mice exposed to elevated CO_2 was significantly lower than in Con females as adults (Table 1), whereas CO_2 exposure starting in adulthood had no effect on body weight (Table 2), suggesting impairment of normal somatic growth, which could also manifest in terms of lung development. It is unclear what the mechanism behind this may be, especially because it was only measured in female mice. Although hypoxia is a well-known cause of intrauterine growth restriction (Kingdom and Kaufmann 1997), our model is far from an *in utero* hypoxia insult, in which oxygen levels are typically considerably lower and CO_2 levels are not necessarily higher. Instead, the greater chord length in CO_2-exposed female mice could be interpreted as impaired alveolarization, rather than alveolar destruction. More detailed probing of lung structure and assessment of parenchymal volume, volume of the alveolar septa, volume of air in the parenchyma, and, most importantly, the number of alveoli in our study

Table 2. Body weight, specific lung function at functional residual capacity, and lung compliance of dams [means ± SDs (n)] exposed to ~890 ppm CO_2 or control (465 ppm) from prepregnancy until 4 wk after giving birth (total ~8 wk).

Measurement	Con	CO_2
Body weight (g)	25.51 ± 1.62 (6)	25.41 ± 1.81 (6)
Lung volume (mL)	0.26 ± 0.03 (6)	0.27 ± 0.02 (6)
sR_{aw} (hPa.s)	0.090 ± 0.012 (6)	0.092 ± 0.014 (6)
sG (hPa)	2.51 ± 0.30 (6)	2.46 ± 0.032 (6)
sH (hPa)	11.15 ± 1.25 (6)	11.13 ± 0.92 (6)
Compliance (mL/cm H_2O)	0.055 ± 0.003 (6)	0.057 ± 0.007 (6)

Note: CO_2, carbon dioxide; Con, control; SD, standard deviation; *sG*, specific tissue damping; *sH*, specific tissue elastance; sR_{aw}, specific airway resistance.

revealed no significant effect of CO_2 exposure in either sex (Table 1). In mice, alveolarization occurs almost entirely postnatally, with new septa forming until at least 5 wk of age (Mund et al. 2008). Although it would seem to make more sense for exposure to elevated CO_2 levels to increase alveolarization so as to increase the surface area for gas exchange (Silva et al. 2015), in our study our measurements showed the opposite in female mice, which had a nonstatistically significant fewer number of alveoli. Male mice, which did not show functional deficits due to CO_2 exposure, had a nonstatistically significant greater alveolar number, consistent with the idea that CO_2 exposure should require a greater lung surface area for gas exchange.

Our model is distinct from others whereby mice exposed to an early life hyperoxic insult display disrupted alveolar development that extends to adulthood (Yee et al. 2006; O'Reilly et al. 2008). Such studies have showed that an early life insult of this sort can have long-term impacts on the lungs. We therefore postulate that exposure to 890-ppm CO_2 impairs lung development in female mice. The mechanism(s) for this are unknown, as are reasons for why this effect was seen only in female mice. Further, we did not measure any change in lung volume in dams exposed as adults, which suggests that alterations in lung volume, structure, and function may be more developmental rather than being an adaptation to CO_2 exposure.

In terms of other statistically significant effects of CO_2 exposure, we also found higher *sH* in female mice exposed to elevated CO_2 (Figure 2D). A higher *sH*, as measured by the FOT, is indicative of increased stiffness of the lung parenchyma (Devos et al. 2017). Greater parenchymal stiffness is a feature of respiratory diseases such as fibrosis (Tanaka et al. 2012). This greater *sH* was accompanied by lower compliance, suggesting a greater effort for ventilation in these mice. To the best of our knowledge, there are no similar data for comparison, with only short-term, high-concentration studies existing which are of little relevance here (Tashkin and Simmons 1972).

Conclusion

The results of our study clearly demonstrate that long-term exposure to a level of atmospheric CO_2 predicted by the most up-to-date climate change models can negatively impact lung structure and function in female mice. Although the effects we saw were modest, there were biologically relevant impairments in a range of lung structure and function parameters. These trends may have progressed to statistical significance if we had continued our exposure for a longer period of time or had a larger sample size. Importantly, our results show that early life is a time that is particularly susceptible to the effects of increased atmospheric CO_2 exposure, with mice commencing exposure as adults showing no adverse effects. Further studies are required to tease out exactly when the most sensitive window of exposure is. Our data suggest that, in this mouse model, the period in which the lung is undergoing rapid growth and alveolarization is particularly important. Regardless, these data suggest that moderate elevations in atmospheric CO_2 cannot be dismissed as insignificant in terms of their direct effects on health. Our data provide the rationale for further exploration of this phenotype. Future research is needed to assess whether long-term exposure to moderately increased CO_2 also negatively impacts other organs that have previously been shown to be impacted by short-term, high-level CO_2 exposure (e.g., the brain, kidneys, and bones). It is our opinion that with atmospheric CO_2 increasing 2–3 ppm/y, it will not be long until a level is reached that is directly detrimental to human health. Thus, continued research in this area and increased effort in curbing CO_2 emissions are both urgently required.

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A.N.L., R.M.L., and C.S.W. discussed the implications of increased atmospheric CO_2 on health and conceived the study. All authors contributed toward study design. A.N.L., M.G.P., and E.K.C. conducted the animal experiments, collected the data, and analyzed the data. L.J.B. processed lung samples and performed stereological analyses. A.N.L. and C.S.W. drafted the manuscript. All authors read the draft manuscript and provided input.

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